Exhibit A

Efficient generation of human T cells from a tissue-engineered thymic organoid

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Biocompatible inorganic matrices have been used to enhance bone repair by integrating with endogenous bone architecture. Hypothesizing that a three-dimensional framework might support reconstruction of other tissues as well, we assessed the capacity of a tantalum-coated carbon matrix to support reconstitution of functioning thymic tissue. We engineered a thymic organoid by seeding matrices with murine thymic stroma. Co-culture of human bone marrow-derived hematopoietic progenitor cells within this xenogeneic environment generated mature functional T cells within 14 days. The proportionate T-cell yield from this system was highly reproducible, generating over 70% CD3⁺ T cells from either AC133⁺ or CD34⁺ progenitor cells. Cultured T cells expressed a high level of T-cell receptor excision circles (TREC), demonstrating de novo T lymphopoiesis, and function of fully mature T cells. This system not only facilitates analysis of the T-lymphopoietic potential of progenitor cell populations; it also permits ex vivo genesis of T cells for possible applications in treatment of immunodeficiency.

Keywords: T lymphocytes, stem cells, hematopoiesis, thymus

Biocompatible materials are commonly used in reconstructive medicine. One such material, CellFoam, has been used to repair bone defects in vivo; the open pore structure permits infiltration and integration of cells into the surrounding engraftment site[1]. CellFoam is fabricated by high-temperature precipitation of tantalum, a highly biocompatible metal with an established history of in vivo medical use[2], as a thin layer onto a highly regular reticulated carbon skeleton. The resulting material is 85–90% porous with a high ratio of surface area to volume (1 cm^3 of CellFoam provides over 600 cm^2 of surface area) and a heavily contoured surface with nanostructure ridges that facilitate cell attachment. Whereas three-dimensionality has obvious advantages for the mechanical needs of bone reconstruction, it is critical for organ tissue function. We therefore tested the ability of the CellFoam matrix to support reconstruction of the thymic microenvironment.

The thymic microenvironment consists of stromal cells (including epithelial cells, dendritic cells, macrophages, and fibroblasts) and the cytokines they elaborate, organized in a three-dimensional infrastructure[3–7]. The complexity of the thymic architecture and cellular composition is such that various developmental niches offer distinct microenvironments adapted specifically for defined stages in T lymphopoiesis.

Several systems currently exist for generating T cells from human CD34⁺ progenitors in vitro[8–18]. However, these systems are often difficult to establish; in addition, some involve the use of recombinant cytokines, and most have a highly variable outcome, yielding only a small number of mature T cells. Reliable quantitation of the T-lymphopoietic capacity of progenitor cells will depend on improvements in these parameters.

Reasoning that the three-dimensional architecture of the thymus contributes to critical cell–cell associations, we cultured thymic stroma on matrices of varying dimensions and pore densities (Fig. 1A). Our goal was to determine the optimum conditions for T lymphopoiesis and to quantify the T-lymphopoietic capacity of human marrow progenitor cells when co-cultured in this xenogeneic environment.

Results

Determination of optimum matrix size and input cell number. The optimal-sized matrix for this system measures 10 mm diameter × 1 mm in depth, with an average pore density of 80 pores per inch (p.p.i.). Poor T-cell production was observed using matrices of smaller or larger dimensions or with different pore densities, regardless of input cell number. Input cell density was also critical for optimum T-cell generation. Using 10 × 1 mm matrices and input cell densities of 1×10^4 progenitor cells per well, $1.9 \times 10^4 \pm 0.63 \times 10^4$ (mean ± s.d.; n = 7) human CD45⁺ leukocytes were generated after 14 days in co-culture. Of these, 78.15 were CD3⁺, representing the predominant cell population. There was no significant increase in the T-cell yield using 1×10^5 input cells (data not shown). No lymphocytes were generated using an input cell density of less than 1×10^4 cells per well. Murine thymic monolayer cultures were substantially less efficient at T-cell generation: only 28.1 ± 5.2% of human CD45⁺ cells generated were CD3⁺, whereas approximately 59.1% were CD14⁺ monocytes (Table 1). The total number of T cells generated on murine stromal monolayers from an input of 1×10^4 progenitors was $4.4 \pm 0.8 \times 10^3$ compared with $15 \pm 0.7 \times 10^3$ generated on thymic stroma established on CellFoam (Table 2).

Immunophenotype of human cells generated in co-culture. The sequential differentiation of T-lymphoid precursors in the co-cultures was tracked by flow-cytometric analysis (Fig. 1B). Nonadherent cells were harvested at the times indicated after establishment of the co-cultures and their immunophenotype determined by FACS analysis. Acquisition of cell surface CD2, CD4, and CD8

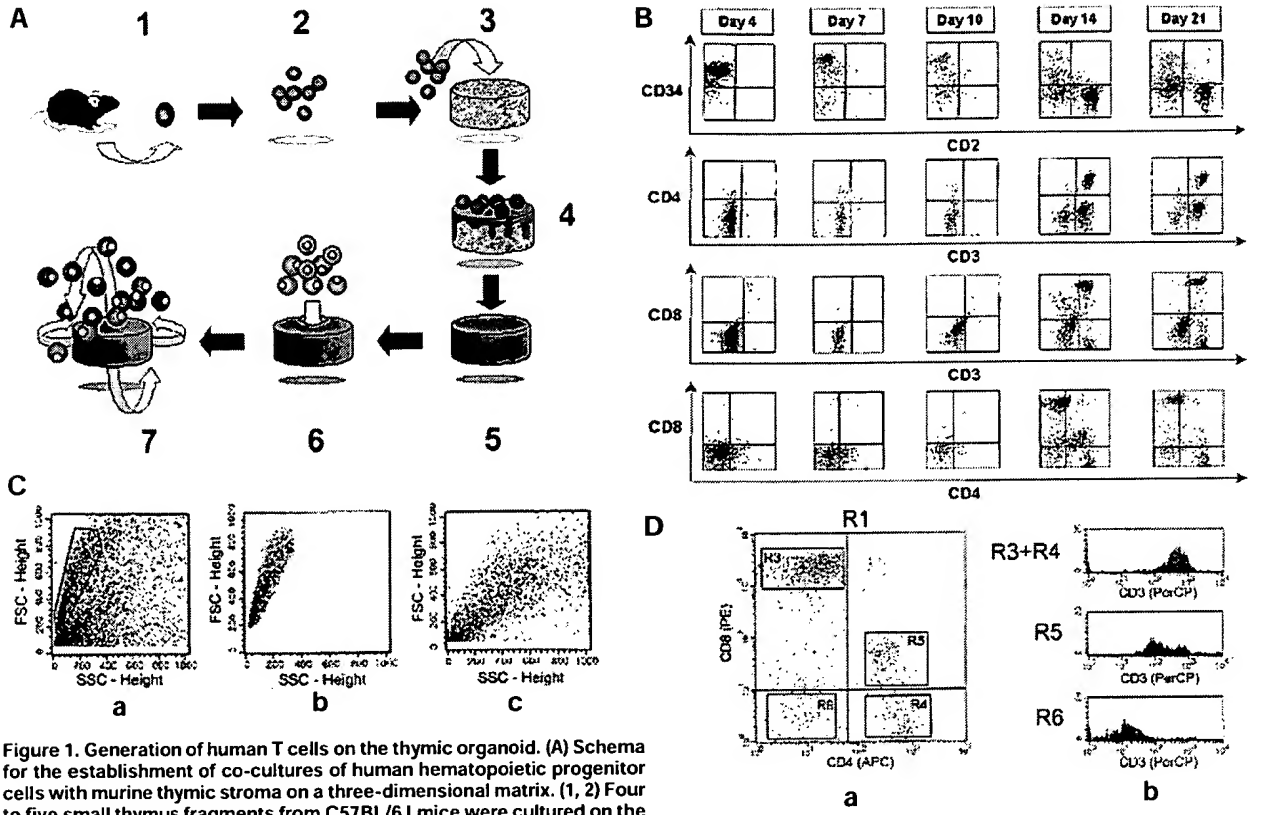

Figure 1. Generation of human T cells on the thymic organoid. (A) Schema for the establishment of co-cultures of human hematopoietic progenitor cells with murine thymic stroma on a three-dimensional matrix. (1, 2) Four to five small thymus fragments from C57BL/6J mice were cultured on the surface of CellFoam disks (3, 4) for 14 days until a confluent layer of stroma had formed throughout the matrix (5). (6) Upon confluence, CD34⁺ or AC133⁺ progenitor cells were added into co-culture. (7) After another 14 days, the cells were harvested. (B) Differentiation kinetics of AC133⁺ bone marrow progenitors in a thymic organoid co-culture system. (C) Light-scatter plots of cells harvested from culture. After 14 days in co-culture, cells were harvested by flushing the wells and matrices with phosphate-buffered saline (PBS). Panel a shows the light-scatter plot from xenogeneic co-cultures. A distinct population of lymphocytes was harvested from the co-cultures (panel b) that was not present in murine thymic cultures alone (panel c). FSC, forward scatter; SSC, side scatter. (D) Differential intensity of CD3 staining on harvested cells. Gating on the lymphocyte population identified in (C). Panel a shows SP CD4⁺, SP CD8⁺, and DP CD4⁺CD8⁺ lymphocytes. The CD3 fluorescence intensity of these cells, and of double-negative (DN) CD4⁻CD8⁻ cells, was shown by gating on cells in each quadrant: R3 = SP CD8⁺; R4 = SP CD4⁺; R5 = DP CD4⁺CD8⁺; R6 = CD4⁻CD8⁻. Panel b shows the CD3 fluorescence intensity of SP, DP, and DN cells. SP cells express CD3 with the highest fluorescence intensity (top); DP cells express intermediate levels of CD3 (middle), and DN cells are CD3⁻ (bottom).

occurred after 14 days in co-culture. Acquisition of CD4 at day 14 was associated with upregulation of CD3. CD3 was co-expressed with the majority of CD8⁺ and virtually all CD4⁺ cells; those cells that were CD3⁻CD8⁺ were found to express TCRvδ (not shown). Discrete populations of single-positive (SP) CD4⁺, SP CD8⁺, and their double-positive (DP) CD4⁺CD8⁺ precursors were evident after 14 days, although the number of DP cells diminished significantly by day 21. After two weeks, the flow-cytometric profile of cells harvested from co-culture revealed a distinct population of cells with forward and side-scatter characteristics of a lymphoid population (Fig. 1C). This population represented 22% of all cells harvested from the cultures, and contained >65% of all human CD45⁺ cells. Over 70% of these lymphoid cells were SP mature T cells, 5.79 ± 0.27% were CD4⁺CD8⁺ DP precursors, <1% were CD56⁺ natural killer (NK) cells, and ~15% were monocytes. DP cells were shown to express intermediate/low levels of surface CD3 (Fig. 1D). The absolute number of human cells was calculated using the percentage of total cells staining with human-specific antibodies (Table 2). T-cell receptor αβ (TCRαβ) was expressed by 80% and TCRγδ by 15% of CD3⁺ SP cells. No lymphoid population was observed in harvests of murine thymic stroma, and murine cells did not stain with the human-specific antibodies used.

To ensure that the T cells recovered from culture were not the product of the small number of contaminating CD2⁺ cells transferred with the AC133 input population, we compared CD2-depleted progenitor preparations with AC133⁺ controls. Mononuclear cells (MNCs) from a single bone marrow sample were divided into two

Table 1. T-lymphopoietic capacity of AC133⁺ and CD34⁺CD2⁻ bone marrow progenitors and percentages of human cells recovered[a]

	A	B	C	D
		Paired comparison (n = 2)		
	CellFoam co-cultures (n = 7)	AC133⁺	CD34⁺CD2⁻	Thymic stromal monolayers (n = 3)
CD3⁺CD4⁺	21.67 ± 4.53	20.05 ± 9.16	20.90 ± 5.69	12.8 ± 1.8
CD3⁺CD8⁺	26.07 ± 14.36	44.60 ± 5.51	46.42 ± 4.85	1.8 ± 0.5
CD4⁺CD8⁺	5.79 ± 0.27	5.98 ± 4.69	6.25 ± 2.51	2.1 ± 0.8
Total CD3⁺	78.15 ± 3.67	70.62 ± 1.03	73.56 ± 1.67	28.1 ± 5.2
CD33⁺	16.15 ± 12.41	N/D	N/D	64.1 ± 7.6
CD14⁺	13.60 ± 4.55	N/D	N/D	59.1 ± 8.3
CD56⁺	0.56 ± 0.12	N/D	N/D	1.7 ± 0.5

[a]Column A shows the percentages of human CD45⁺ cells recovered. AC133⁺ (column B) and CD34⁺CD2⁻ (column C) progenitors isolated from the same source of bone marrow MNCs were placed in independent co-cultures at the same cell density (1 × 10⁴ cells per well). For comparison, 1 × 10⁴ progenitors placed in co-culture with murine thymic monolayers (column D) generated fewer T-lymphoid cells, but a higher percentage of monocytes (see Results section).

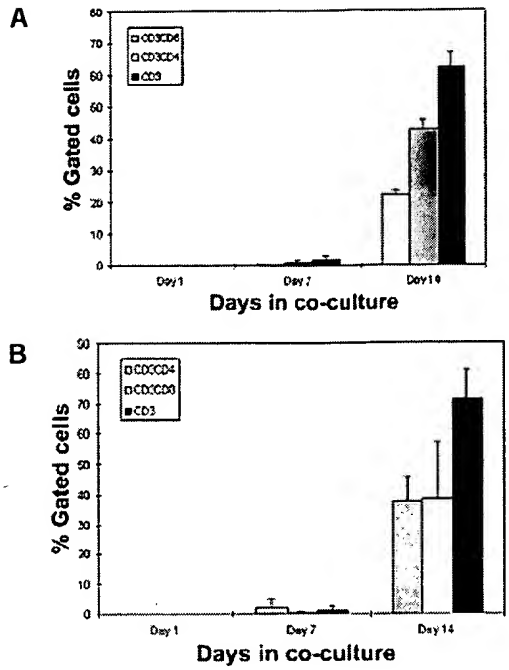

Figure 2. (A) Intrasample variability. Multiple co-cultures were established using a single source of AC133$^+$ cells at fixed cell densities on separate matrices impregnated with murine thymic stroma. Variation in generation of T lymphocytes was analyzed by cell count using Trypan Blue exclusion and immunophenotypic analysis. The mean percentage ± s.e.m. for each of the CD3$^+$, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ subpopulations of T cells is shown for each time point in the co-culture, from three independent experiments. (B) Intersample variability. A fixed number of cells from a distinct source of CD34$^+$ cells were introduced into co-culture, and after 7 and 14 days in co-culture, the immunological characteristics were analyzed. The mean percentage ± s.e.m. for each of the CD3$^+$, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ subpopulations of T cells is shown for each time point, from seven independent experiments.

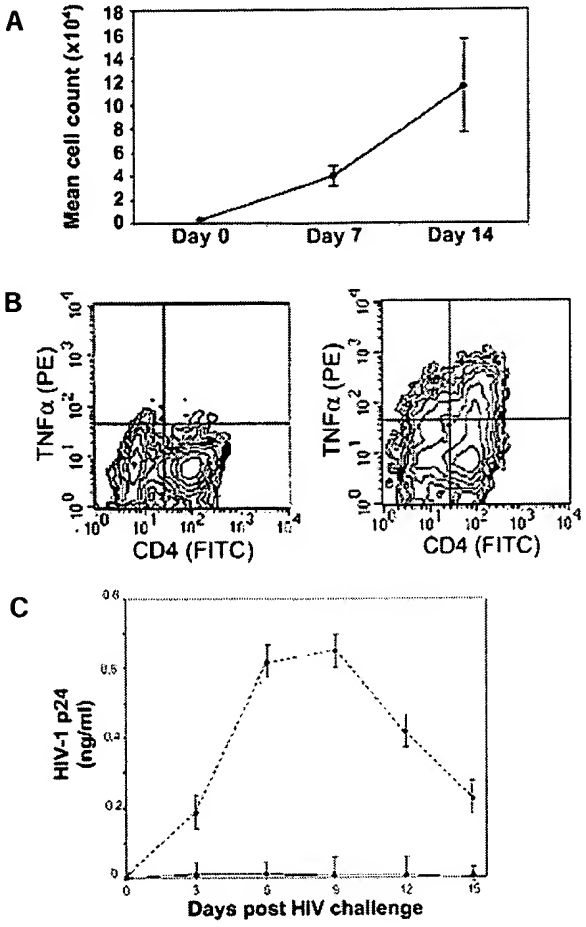

Figure 3. (A) T-cell proliferation in response to mitogenic stimulation. T cells generated in vitro were removed from co-culture and incubated for seven days in the presence of IL-2 (10 IU ml^{-1}) and PHA (2 μg ml^{-1}). Cells were counted and analyzed by flow cytometry. The percentage increase in CD3$^+$ cells is presented from three independent experiments. (B) Cytokine production in response to mitogen stimulation. T cells were harvested from culture and stained with immunofluorescent antibodies to TNF-α before and 6 h after stimulation with Con A at a concentration of 5 μg ml^{-1}. Stimulation resulted in a marked increase in the level of TNF-α (panel b) over the baseline level (panel a), as detected by flow cytometry. (C) HIV-1 proliferation in CD3$^+$CD4$^+$ cells generated from human hematopoietic progenitor cells on murine thymic stroma. CD3$^+$CD4$^+$ cells generated from human hematopoietic progenitor cells on murine thymic stroma were challenged with a T-cell tropic strain of HIV, HIV-1$_{IIIB}$. HIV-1 p24 antigen was then quantified in the supernatant of cultures at 3, 6, 9, 12, and 15 days following viral challenge. HIV-1 p24 production following HIV-1 challenge (solid line) or challenge with heat-inactivated HIV-1 (dashed line) are shown.

equal aliquots. Progenitor cells from one fraction were isolated by immunomagnetic selection of AC133$^+$ cells; MNCs in the other fraction were immunomagnetically selected on the basis of CD34 expression, and cells from this second fraction were subsequently depleted of CD2$^+$ cells. Cells were harvested after 14 days and stained for immunophenotypic analysis. Human cells were distinguished by expression of CD45. The T-cell yield was virtually identical with or without CD2$^+$ depletion (70.62 ± 1.03% and 73.56 ± 1.67% CD3$^+$ cells from the AC133$^+$ and CD34$^+$CD2$^-$ populations, respectively) (Table 1), indicating the lack of contribution from contaminating CD2$^+$ cells.

TCR excision circle (TREC) assay. To assess de novo generation of T cells and discriminate it from simple expansion of contaminants, the TREC assay was performed. The ratio of TREC to β-actin from T cells generated in vitro (0.016) was equivalent to that of human fetal thymocytes (0.017), suggesting a comparable rate of new T-cell generation. Interestingly, a discrete population of cells harvested from the periphery of a co-culture plate was found to contain >95% CD3$^+$ cells, and the ratio of TREC to β-actin for these cells was appreciably higher (0.54). The ratios of TREC to β-actin from peripheral blood monocytes (PBMCs) and from AC133$^+$ bone marrow mononuclear cells was significantly lower (Table 3). No TREC was detected in any of the samples of B cells tested or from murine thymic stroma.

Intra- and intersample variability in numbers of T cells generated. To determine the variation of T-cell output within a given source of progenitors, we established multiple co-cultures using a single source of AC133$^+$ cells at fixed cell densities (1×10^4 cells per well) on separate 10×1 mm matrices of murine thymic stroma. Within each sample, variation of T lymphocytes generated was analyzed by cell count using Trypan Blue exclusion, and by immunophenotypic analysis. Human cells were distinguished by surface staining with human-specific anti-CD45. After seven days in co-culture, the number of mature T cells detected was low: CD3$^+$ cells represented 2.02% (mean ± s.e.m.; n = 3) (CD3$^+$CD4$^+$ 1.0 ± 0.52% and CD3$^+$CD8$^+$ 0.58 ± 0.1%) of the CD45$^+$-gated population. However, by 14 days, the proportion of CD3$^+$ cells rose to 62.16 ± 4.53%; and the percentages of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells were 42.7 ± 2.87% and 22.39% ± 1.29%, respectively (Fig. 2A).

From sample to sample, variation was calculated by comparing the proportion of T cells generated from separate sources of $CD34^+$ progenitors, again using a fixed number of progenitor cells (1×10^4 cells per well). After 14 days 71.21 ± 9.87% of the cells were $CD3^+$, 37.44 ± 8.44% $CD3^+CD4^+$, and 38.06 ± 19.13% $CD3^+CD8^+$ (Fig. 2B). These data demonstrate a high level of reproducibility within the system that suggests its potential in comparative analyses of input populations.

Analysis of Vβ TCR repertoire. T cells generated in the co-culture system demonstrated a diverse TCR Vβ expression as determined by reverse transcription–polymerase chain reaction (RT-PCR). TCR Vβ expression among human T cells generated in the co-culture compared favorably to peripheral blood T cells, with 13 of 24 TCR Vβ families represented, compared with 14 of 24 in controls. TCR Vβ expression was not detectable by this method in $CD34^+CD2^-$ cells before co-culture or from lysates of murine thymic stroma alone (data not shown), arguing against a contaminating population of T cells contributing to the T-cell output.

Functional analysis of T cells generated in vitro. The ability of cells to respond to mitogenic stimuli was assessed as one indication of functional capability. Cells removed from co-culture after 14 days showed pronounced proliferation when placed in liquid culture with complete medium plus interleukin 2 (IL-2; 10 IU ml^{-1}) and phytohemagglutinin (PHA; 2 μg ml^{-1}). After an additional seven days in culture there was a 45-fold increase in cell number (Fig. 3A). Of these cells, >90% were $CD3^+CD4^+$ TCR $\alpha\beta^+$; 3% were $CD3^+CD8^+$ TCR β^+, and 3% β^+. No cells expressing TCRγδ were detected. In addition, T cells generated in vitro responded to concanavalin A (Con A) stimulation by producing intracellular cytokines comparable to levels in fully mature peripheral blood T-cell controls. Intracellular tumor necrosis factor α (TNF-α) levels are shown in Figure 3B.

To further assess functional characteristics of cells, we exposed them to T-cell restricted strains of HIV-1. $CD3^+CD4^+$ T cells generated in co-cultures were infectable by $HIV\text{-}1_{IIIB}$, as demonstrated by HIV-1 p24 antigen production following challenge with virus to a peak of 0.6 ± 0.05 ng ml^{-1} (Fig. 3C). No HIV-1 p24 antigen production was demonstrable in $CD3^+CD4^+$ T cells generated in co-cultures that were challenged with heat-inactivated virus (Fig. 3C) or in cultures of human $CD34^+CD2^-$ input cells or murine thymic stroma exposed to infectious $HIV\text{-}1_{IIIB}$.

Discussion

This study demonstrates that human T cells efficiently differentiate from progenitor cells in the context of a three-dimensional matrix on which murine thymic stroma has been cultured. Whereas T-cell production was more efficient in three dimensions than in a monolayer culture system, the precise benefit provided by the three-dimensional structure is uncertain. Because the chemical composition of the matrix precluded histologic section, any hypothesis regarding whether complex microenvironments of the thymus structure were reconstituted could not be addressed. Analysis of stromal cell subsets and selective addition or subtraction of cellular elements will permit definition of the specific stromal cell types critical for T-cell genesis.

Estimates vary as to the time required for a progenitor cell to differentiate into a fully mature T cell within the thymus. However, our observations were comparable to studies demonstrating that within a three-dimensional microenvironment this process takes about two to three weeks[19,20].

Immunophenotypic analysis of fresh thymocytes represents a longitudinal view of T lymphopoiesis with cells at multiple stages of maturation. In contrast to the situation with fresh natural thymus, we have not demonstrated a stage at which DP cells predominate. We hypothesize that this reflects differences in the dynamics of T-cell differentiation rather than substantial differences in ontogeny. Since in our in vitro system the "thymus" is colonized by a single cohort of progenitors that mature in concert, rapid transition through the DP stage may mean that only a small number of DP cells exist at any given time. In addition, accumulation of mature T cells within an enclosed system will also reduce the ratio of DP to SP cells.

Although a small number of $CD2^+$ cells were transferred into our co-cultures with the $AC133^+$ progenitors, our data indicate that the T cells generated were not derived from these contaminating cells. We, and others[16], have observed that the deliberate introduction of mature human T cells into co-cultures does not result in increased numbers of T cells or their precursors, and we noted no change in output with highly purified, $CD2^-$ progenitor cells.

The high level of TREC observed in the cells harvested from co-culture demonstrated conclusively that these T cells were generated de novo from progenitor cells. The TCRvδ locus is excised during TCRα *VD-J* rearrangement, forming a TREC[21,22]. Since TRECs are stable[23] and do not duplicate when the T cell divides[24], TREC levels are highest in recent thymic emigrants and decline exponentially as cells undergo division. Quantitation of TREC has been shown to be a reliable tool for monitoring de novo T-cell generation[25]. The 1,000-fold increase in TREC we observed after 14 days in co-culture could not be the product of proliferating contaminants and demonstrate T-cell neogenesis. The high level of TCRαβ versus TCRγδ T cells suggests that the T-cell generation occurring mimics that of intrathymic rather than extrathymic differentiation, and the abundance of TREC was comparable to primary human thymus.

Several unexpected features of the system were observed. The failure to generate T cells at input cell densities of fewer than 1×10^4 progenitors may represent a critical dilution of the T-cell progenitor or loss of cell–cell interactions at low cell concentrations. In addition, the dimensions of the matrices required for successful T-cell neogenesis were shown to be critical. Miniaturizing the system by

Table 2. Comparison of two- versus three-dimensional murine thymic co-culture technique[a]

Immunophenotype	Number of cells generated × 10^3 (mean ± s.d.) 3D (n = 7)	2D (n = 3)
Total $CD3^+$	15.0 ± 0.7	4.4 ± 0.8
$CD3^+CD4^+$	4.2 ± 0.9	2.0 ± 0.1
$CD3^+CD8^+$	5.0 ± 2.8	0.3 ± 0.1
$CD33^+$	1.1 ± 0.1	0.3 ± 0.1
$CD14^+$	3.1 ± 2.4	10.0 ± 1.2
$CD56^+$	0.1 ± 0.2	0.3 ± 0.1

[a]Number of cells generated from 1×10^4 hematopoietic progenitors. $AC133^+$ progenitors were co-cultured with murine thymic stroma on a CellFoam biomatrix (3D) xor monolayers of murine thymic stroma (2D). The number of human $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD8^+$, $CD33^+$, $CD14^+$, and $CD56^+$ cells was calculated using the percentage of each immunophenotype by human-specific staining as applied to the total number of cells harvested at day 14 of the co-culture.

Table 3. Quantification of TREC by PCR[a]

Source	n	Ratio of TREC to β-actin
Murine thymic stroma	7	0
Human B cells	7	0
Human bone marrow $AC133^-$ MNCs	3	0.000014
Adult human peripheral blood MNCs	3	0.0014
Human fetal thymocytes	3	0.017
T cells from in vitro assay	2	0.016

[a]The mean ratio of TREC to β-actin is shown for murine thymocytes, human B cells, bone marrow $AC133^+$ progenitor cells, adult human peripheral blood MNCs, fetal thymocytes, and T cells produced from $AC133^+$ cells after 14 days in co-culture with murine thymic stroma on CellFoam.

using smaller matrix sizes and input cell densities failed to generate T cells. We can only speculate that critical thresholds for selected stromal elements were not reached, specific cell–cell relationships were not optimized, or nutrient exchange conditions were affected by matrix and pore size.

The characteristics of this system, particularly the low intra- and intersample variability, present unique opportunities to assess the lymphopoietic capacity of hematopoietic progenitors, either as distinct subsets from different tissues or disease states, or following specific manipulations. In addition, the system shows promise for investigations of human T-cell selection processes for ex vivo generation of antigen-reactive cells.

Experimental protocol

Murine thymic stromal cultures on three-dimensional matrices. Thymus obtained from freshly sacrificed four- to six-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), at least 48 h post shipment, in accordance with institutional review board guidelines, was separated into fragments <0.5 mm^3 and five to six fragments applied either directly to the surface of disks of CellFoam (Cytomatrix, Woburn, MA) or to tissue culture plates in the absence of CellFoam. Matrices of five different dimensions (5 mm diameter × 1 mm height, 5 × 2 mm, 10 × 1 mm, 10 × 2 mm, and 30 × 2 mm) and three different pore densities (60, 80, or 100 p.p.i.) were tested. All matrices were cultured in 24-well tissue culture plates, except the 30 × 2 mm matrices, which were cultured in 6-well tissue culture plates. Complete medium, consisting of Iscove's modified Dulbecco's medium (IMDM; Mediatech, Washington, DC) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO), glutamine (1 mM), penicillin (10 IU ml^{-1}), and streptomycin (10 mg ml^{-1}), was added to totally immerse the matrices. Medium was changed every four days. All cultures were incubated at 37°C in 5% CO_2.

Murine thymic stromal cultures on CellFoam were ~80% confluent, with stromal elements seen through all levels of the matrix by day 14, as determined by phase-contrast light microscopy. In the absence of CellFoam, murine thymic stroma had formed a 60–80% confluent monolayer after 14 days. At this time the colonized CellFoam was transferred into fresh culture plates and cultured for an additional 24 h in complete medium before adding progenitor cells.

Purification of human bone marrow-derived AC133$^+$ or CD34$^+$ progenitor cells. Bone marrow was obtained by posterior iliac crest aspiration from healthy adult volunteers in accordance with institutional review board guidelines. Following density gradient centrifugation (Ficoll-Paque; Pharmacia Biotech Inc., Piscataway, NJ), AC133$^+$ mononuclear cells (MNCs) were isolated using an AC133 Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, CA). More than 98% of the selected AC133$^+$ cells were CD34$^+$ and only 0.57 ± 0.29% (mean ± s.e.m.; n = 7) were CD2$^+$. CD34$^+$ cells were isolated using a CD34 Multisort Kit (Miltenyi Biotec) that allowed subsequent depletion of CD2$^+$ cells. No CD2$^+$ cells were detected by flow cytometry following CD2 depletion. Using both methods of progenitor cell isolation >90% of CD34$^+$ cells co-expressed CD38, with approximately 5% being of the more immature CD34$^+$CD38$^-$ immunophenotype.

Assay of T-lymphopoietic capacity of human bone marrow progenitor cells. AC133$^+$ cells were added to the thymic stromal cultures at cell densities of 1×10^5, 1×10^4, or 1×10^3 cells per well. CD34$^+$CD2$^-$ cells were added at a density of 1×10^4 cells per well. Nonadherent cells were harvested by gentle aspiration at 4, 7, 10, 14, and 21 days, counted and assessed by flow cytometry using fluorochrome-conjugated antibodies against TCRαβ, TCRγδ, CD2, CD3, CD4, CD8, CD14, CD33, CD34, and CD56 and isotype controls (Becton Dickinson, San Jose, CA). Analysis of the immunophenotype of cells cultured was performed using a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software.

T-cell proliferation in response to mitogen, cytokine production, and HIV-1 infectablity. Proliferative responses of T cells generated in co-culture were measured using $3.0–3.5 \times 10^3$ cells ml^{-1} with 10 IU ml^{-1} IL-2 and 2 μg ml^{-1} PHA in 24-well plates. After seven days, cells were counted and analyzed by flow cytometry. To evaluate cytokine production by unstimulated and stimulated T cells generated in co-culture, 150,000 cells per well were stimulated with Con A (Sigma; final concentration of 5 μg ml^{-1}), treated with Brefeldin A (Sigma), harvested, and stained with anti-CD4 and anti-CD8, followed by intracellular staining for TNF-α (Caltag, Burlingame, CA).

Cell-free supernatants of live or heat-inactivated HIV-1_{IIIB} were added at an infectious dose of 100 ng HIV-1 p24 antigen per ml to 1×10^5 nonadherent cells harvested from 14-day co-cultures. Cells harvested from cultures of murine thymic stroma alone and CD34$^+$CD2$^-$ cells were also challenged with HIV-1_{IIIB} as above. Supernatants were harvested from the cultures at three-day intervals and the HIV-1 p24 antigen measured by ELISA (DuPont, Boston, MA).

Analysis of Vβ TCR expression and quantitation of TCR α1 TRECs with real-time PCR and molecular beacon technology. Vβ TCR expression in T cells generated in the co-culture was determined according to an established methodology[26]. cDNA generated from 1×10^5 PBMCs and 1×10^5 CD34$^+$CD2$^-$ cells were also tested for Vβ TCR RNA expression. A total of 24 sets of primers specific for Vβ TCR RNA were used. Negative controls contained distilled water alone.

TREC analysis was performed on cells derived from each co-culture set. DNA extracted from 1×10^4 to 1×10^6 cells (QIAamp blood kit; Qiagen, Valencia, CA) was resuspended in 200 μl of double-distilled water (ddH_2O). Final DNA concentrations ranged from 15 to 315 ng μl^{-1} and all samples typically had A_{260}/A_{280} ratios above 1.6. Polymerase chain reaction was performed in 50 μl reactions containing 5 μl of the DNA prep, 1× TaqMan Buffer A, 3 mM $MgCl_2$, 200 μM each of dATP, dCTP, and dGTP, 400 μM dUTP, 300 nM of each primer, 100 nM probe, 0.025 U μl^{-1} AmpliTaq Gold, and 0.01 U μl^{-1} AmpErase UNG (all from PE Biosystems, Foster City, CA). Temperature cycling on an ABI 7700 began with two initial holds at 50°C for 2 min and 95°C for 10 min; these were followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. All samples were run in triplicate, including standards and controls.

The primer and probe sequences were as follows:

Sense:	TREC5: 5′-CATCCCTTTCAACCATGCTGACACCTCT-3′
Antisense:	TREC3: 5′-CGTGAGAACGGTGAATGAAGAGCAGACA-3′
Sense:	β-actin5: 5′-TCACCCACACTGTGCCCATCTACGA-3′
Antisense:	β-actin3: 5′-CAGCGGAACCGCTCATTGCCAATGG-3′

The following probes were labeled with a 5′ reporter dye (specified below) and a 3′ quencher dye, TAMARA (PE Biosystems). The sequences were as follows:

TREC:	5′-VIC-TTTTTGTAAAGGTGCCCACTCCTGTGCACGGTGA-3′
β-actin:	5′-FAM-ATGCCCTCCCCCATGCCATCCTGCGT-3′

Standards for both TREC and β-actin quantification were included in every PCR reaction. To generate the TREC standard curve, primers from Douek and coworkers[25] were used to amplify a 376 bp fragment, which was cloned into the pAMP1 plasmid (Life Technologies, Rockville, MD). The TREC standard curve included eight fivefold dilutions of TREC plasmid spanning from 10 copies to 1×10^5 copies. Likewise, DNA was amplified with the above β-actin primers and cloned into pAMP1. A β-actin standard curve was generated using seven 10-fold dilutions spanning 1×10^1 to 1×10^7 copies. As negative controls, amplification of DNA from B-LCL and ddH_2O (no template) was included in every run. DNA extracted from HIV-seronegative subjects with known TREC levels was also included in every run.

The corresponding cycle threshold values for TREC and β-actin were converted to DNA copy numbers, and the ratio of TREC to β-actin was calculated (Table 3). A recent study commented that β-actin was potentially confounding because of the presence of pseudogenes[27]. However, an analysis of differing cell numbers and cell types (B-LCL, T-cell clones, thymocytes, PBMCs) from at least 10 individuals showed an extremely high correlation between cell input number and β-actin copy number ($R = 0.9$). This indicated that most individuals have similar numbers of pseudogenes capable of amplification with this primer set and therefore validates their use as a standard for cell input number.

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